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Purpose and scope: we are conducting a large case-control study, nested within a prospective cohort, to estimate relative risk of breast cancer in post-menopausal women by levels of urinary estrogens and estrogen metabolites, and to examine the association between the concentrations of these hormones and breast cancer risk with polymorphic variations of a series of candidate genes, known to be implicated in the regulation of estrogen metabolites synthesis.

Progress report: in this first year of the project, cases and matched control subjects were identified, and their urinary samples retrieved from the biorepository in Utrecht (the Netherlands). After centrifugation of the urine samples to prepare the pellets for DNA analysis, cases and matched controls were sent to Dr. Kurzer's laboratory (University of Minnesota, USA) for hormonal analyses by gas chromatography/mass-spectrometry. The laboratory of Dr. Kurzer had been prepared, and her personnel hired for running these analyses.

Conclusions: our study started without problems, and is mostly on schedule. Urinary DNA has been extracted and prepared for genotyping of polymorphic variants in CYP1A1, CYP1B1, CYP3A4 and COMT genes, which will be measured in year 3 of the project. Measurements of estrogen metabolites will start in September/October 2003.

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INTRODUCTION:

It has long been recognized that estrogenic steroid hormones, particularly 17 β-estradiol (E_2) can promote the development of breast tumors. Besides stimulating cell proliferation, there is increasing experimental evidence that estrogens may also be activated into genotoxic hydroxy metabolites that cause DNA mutations. In addition, some of the same metabolites may bind irreversibly to estrogen receptors, and thus stimulate cell proliferation permanently.

Major pathways through which hydroxy metabolites of estrogens (estrone [E_1] and estradiol [E_2]) are formed are the 16α-hydroxylation pathway – which leads to formation of 16α-hydroxy E_1 and estriol – and pathways that lead to 2- and 4-hydroxy ("catechol") estrogens. Preliminary epidemiological evidence suggests that estrogen metabolism via the 16α-hydroxy pathway is increased in breast cancer patients compared to controls, and an inverse relationship has been found between breast cancer risk and the ratio of urinary concentrations of 2-hydroxy/4-hydroxy or 2-methoxy/4-methoxy estrogens.

Among key enzymes involved in the natural conversion of estrogens to hydroxy estrogens are CYP1A1, CYP1B1, and CYP3A4. Furthermore, catechol-O-methyl transferase (COMT) is a key enzyme in the methoxylation of 2- and 4- hydroxyl groups, thus leading to methoxy estrogens. Methoxylation is a major pathway for the inactivation of the very reactive chemicals catechol estrogens. In addition, experimental studies indicate that the methoxy metabolites inhibit tumor formation and development by decreasing cell growth, and inhibiting the formation of blood vessels in tumors.

Given these various observations, it has been hypothesised that breast cancer risk would be lower in women who produce more of 2- and 4-metyhoxy estrogens relative to the levels of corresponding hydroxyl estrogens. To examine the above hypotheses, we have started a case-control study nested within a large prospective cohort (the 'DOM' cohort, the Netherlands), with the following specific aims:

1) examine relationships of post-menopausal breast cancer risk with absolute and relative prediagnostic urine levels of 2-hydroxy, 4-hydroxy, 16α-hydroxy, 2-methoxy and 4-methoxy metabolites of E_1 and E_2
2) examine relationship of polymorphic variants of genes encoding estrogen-metabolizing enzymes (CYP1A1, CYP1B1, CYP3A4 and COMT) to urinary levels of the various estrogen metabolites, as well as to breast cancer risk.

Our project is designed as a case-control study nested within a large prospective cohort, using urine and DNA samples collected from more than 50,000 women in the Dutch city of Utrecht and surroundings ("DOM" cohort). This cohort is unique, in that large volumes (50-100 ml) of urine were collected and stored for all study subjects. The majority of women in the cohort provided also a second (and even third) urine sample. The samples were stored in a large warehouse at -20^0C. Relatively large volumes of urine (>10 ml) are needed to measure the estrogen metabolites, by gas chromatography coupled with mass spectrometry (GCMS). Cases and controls are selected among women who were post-menopausal at recruitment, and who did not use hormone replacement therapy. We also incorporated into our study a

second urine sample for about 60% of women who provided a second urine sample within a time interval of about one year, so as to improve exposure measurements.

BODY:

For year 1, our work plan was (as in the "Statement of Work" of the original grant application):

1) Selection of cases (>300) and controls (equal number), using the established eligibility and matching criteria, and extraction of case-control data sets with relevant information from questionnaires and anthropometry: **Task 1 (months 1-2)**
2) Retrieval of urine samples from the "DOM" repository; centrifugation of urine samples to prepare pellets cellular sediments from which DNA can be extracted; assembly of matched case-control sets for urinary analyses of estrogen metabolites (work subcontracted to the University of Utrecht): **Task 2 (months 1-6)**
3) Start of the analyses of urinary measurements of estrogen metabolites by GC/MS (work subcontracted to the University of Minnesota): **Task 3 (months 3-12)**
4) Extraction of DNA from cell pellets; shipment of extracted DNA to IARC for measurement of genetic variants. Work subcontracted to the University of Utrecht: **Task 4 (months 9-12)**

Tasks 1, 2 and 4 were fully met, whereas task 3 has had some delay:

Task 1: In September 2002, a total of 374 post-menopausal breast cancer cases were identified in the "DOM" cohort. The cases were eligible for the study if the diagnosis of breast cancer was done after the donation of the urinary samples. Of the 374 cases, 355 had at least two urine samples, 350 had two urine samples collected at a time interval of less than 2 years, and 21 cases only had a third urine sample collected. We therefore decided to work with subjects who had two urine samples collected at a time interval of less than 2 years. Among the 350 selected cases, we identified 324 cases who had a natural menopause (defined as no menstruation for the last 12 months and no surgical intervention), were not using hormone replacement therapy and had no prior history of breast cancer at the time of collection, and therefore eligible for the study. We subsequently identified the control subjects, defined as women who were alive and free of any cancers at time of diagnosis of the case, who had natural menopause, who were not using hormone replacement therapy, and who matched the cases for age and date of entry in the cohort. For the 324 selected cases and the 324 matched controls, detailed questionnaire information on smoking, previous use of hormones replacement therapy, anthropometry, reproductive and menstrual history are available.

Table 1. Selected characteristics[1] of study subjects at cohort baseline (round 1), and after 1-year follow-up (round 2)

	Cases (n=324)	*Controls (n=324)*	*P diff.*
Age at blood donation round 1 (years)	58.7 (53.0-64.0)	58.6 (53.0-64.0)	-
Time interval between urine samples (months)	12.5 (11.7-15.6)	12.5 (11.7-15.6)	-
Height round 1 (cm)	162.4 (153.0-173.5)	162.0 (152.5-171.5)	0.43
round 2 (cm)	162.2 (152.5-172.5)	161.8 (152.0-171.5)	0.36
Weight round 1 (kg)	70.8 (54.0-90.5)	69.3 (54.5-90.5)	0.10
round 2 (kg)	71.0 (55.0-90.5)	69.3 (55.5-89.5)	0.05
BMI round 1 (kg/m^2)	26.8 (21.6-34.7)	26.5 (20.4-33.8)	0.25
round 2 (kg/m^2)	27.0 (21.0-34.1)	26.5 (20.7-33.8)	0.18
Age at first full term pregnancy [2]	27.4 (21.0-35.0)	27.5 (20.0-36.0)	0.78
Number of children [2]	3.0 (1.0-6.0)	3.2 (1.0-7.0)	0.51
Ever full-term pregnant[3]	81.2%	77.5 %	0.27
Time since menopause (years)	9.0 (2.0-18.0)	9.1 (2.0-18.0)	0.67
Age at menopause (years)	49.7 (42.0-55.0)	49.5 (42.0-55.0)	0.7
Family history of breast cancer	16.1%	9.5%	0.02
Use of O.C.[3]	4.9%	2.8%	0.17

[1] Mean and 5th and 95th percentiles for continuous variables
[2] Among parous women only
[3] Ever *vs* never

Task 2: From October 2002 to December 2002, urine samples from the 324 cases and 324 matched controls were identified. For each subject, two urine samples (collected about 1 year apart) were selected. Selection criteria of cases and controls were discussed by regular email and telephone contacts between scientists of the International Agency for Research on Cancer (Lyon, France) and scientists of the University Medical Center Utrecht (UMCU) (The Netherlands). A meeting was held at the end of September 2002, in Utrecht (the Netherlands) to determine the final study population.

All urine samples of the "DOM" cohort are stored with an identification number in a commercial freezing storage in Bunnik, 8 kilometers from the UMCU, in large metal pallets of up to three meters high. Two persons are needed for the retrieval of the samples, and are not allowed to stay in the room at -20^0C for more then half an hour.

For the current project, the retrieval of all urine samples was done from October 2002 to January 2003, and samples of the cases and matched controls were retrieved and handled at the same time. The retrieval was done in three subsets of 120 cases and 120 controls, and after the centrifugation of urine samples to prepare pellets for DNA analyses, each subset was shipped to the laboratory of Dr. Kurzer (University of Minnesota, St. Paul, Minnesota, USA), between January and March 2003.

Task 3: The measurement of urinary estrogen metabolites will be performed by the laboratory of Dr Mindy Kurzer, University of Minnesota, using gas-chromatography coupled with mass-spectrometry. A collaborative research agreement was prepared, between IARC and Dr Kurzer's research department, for the transfer of funds needed to make these measurements (subcontract to Dr Kurzer).

In practice, this caused some delay in the money transfer (IARC could not issue the agreement before receipt of the grant, and Dr Kurzer could not start working before receiving her funds). Additional delay was caused by some internal re-organization of personnel in Dr. Kurzer's laboratory. The postdoc and a laboratory technician that were in charge of the estrogen metabolite measurements in Dr Kurzer's laboratory left, and needed to replaced by new personnel. Using the funds of her subcontract, Dr Kurzer hired a new technician, who finally started in June 2003 to work full-time on the estrogen metabolite measurements for our project. This technician has now been fully trained to run the GCMS method measurements and will start measurements in the DOM urine samples in September/October this year. Dr Kurzer plans to hire a second technician, so as to accelerate the pace of measurements and recuperate the time lost during last several months of year 1.

Task 4: From January 2002 to April 2003 DNA was manually extracted from the sediments (cellular pellets) obtained by centrifugation of the urine samples. DNA of 648 women is ready for shipment to IARC for measurement of genetic polymorphisms in the CYP1A1, CYP1B1, CYP3A4 and COMT genes (planned in year 3).

KEY RESEARCH ACCOMPLISHMENTS

Key accomplishments in year 1 were:

- a full selection of post-menopausal breast cancer cases and matched controls within the large prospective "DOM" cohort;
- retrieval of the urine specimens of the breast cancer and controls, and shipment of the samples to Dr. Kurzer's laboratory for measurements of estrogen metabolites;
- preparation of Dr Kurzer's laboratory, to start the urinary analyses of estrogen metabolites by gas chromatography/mass spectrometry in September/October 2003; and
- extraction of urinary DNA for all cases and matched controls.

REPORTABLE OUTCOMES

There are no reportable outcomes so far.

CONCLUSIONS

Our study started without problems, and is almost on schedule. Measurements of urinary estrogen metabolites will start by September/October 2003, and will require about 1.5 years to be completed. DNA has been extracted and prepared for genotyping of polymorphic variants in the CYP1A1, CYP1B1, CYP3A4 and COMT genes, which will be measured in year 3 of the project.

REFERENCES

None.

APPENDICES:

None.